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Uses of knockout, knockdown, and transgenic models in the studies of glucose transporter 4

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Abstract

Currently, glucose transporter 4 (GLUT4) has been considered as the key player for the insulin-stimulated glucose transport in the muscle and adipose tissues. The development of recombinant DNA techniques allows the creations of genetically knockout, knockdown and transgenic animals and cells for the study of GLUT4's physiological functions. Here, we have used key words to search the PubMed and summarized the methods used in *Slc2a4* gene knockout, GLUT4 knockdown and overexpression in the whole body and tissue specific manner. The whole body GLUT4-null mice have growth retardation, but normal glucose tolerance and basal glucose turnover rates. Compared with whole body *Slc2a4* knockout mice, adipose and muscle double knockout mice have impaired insulin tolerance and glucose intolerance. The results of GLUT4 knockdown in 3T3-L1 adipocytes have shown that its expression is needed for lipogenesis after, but not during, differentiation. Transgenic mice with the whole body GLUT4 overexpression have normal body weight and lowered blood glucose level. The adipose tissue specific overexpression of GLUT4 leads to increases in mouse body weight and adipose tissue weight. The insulin-stimulated GLUT4 translocation in the skeletal muscle contributes to the regulation of glucose homeostasis. Data from both transgenic overexpression and tissue specific *Slc2a4* knockout indicate that GLUT4 probably plays a role in the glucose uptake in the fasting state. More studies are warranted to use advanced molecular biology tools to decipher the roles of GLUT4 in the control of glucose homeostasis.

Key Words: Glucose transporter 4; Knockout; Knockdown; Transgene; Overexpression; Insulin

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Core Tip: The whole body glucose transporter 4 (GLUT4)-null mice have growth retardation, but normal glucose tolerance and basal glucose turnover rates. The muscle-specific GLUT4 knockout mice have normal body weight and fat pad weight at least before 6 mo of age, whereas the adipose-GLUT4 knockout mice have glucose intolerance. The adipose and muscle GLUT4 double knockout mice develop hyperglycemia in the fasting state, suggesting the role of GLUT4 in fasting state. Compared to the control mice, wholebody GLUT4 transgenic mice have similar growth rate before 10 wk of age, lower blood glucose in the fasting, and lower insulin level in the fed state. The adipose tissue specific GLUT4 overexpression increases body weight, glucose transport rate and adipose tissue weight. Data from both transgenic overexpression and tissue specific knockout of GLUT4 indicate that GLUT4 probably plays a role in the glucose uptake in the fasting state.

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INTRODUCTION

Genes in an organism are codes responsible for genetic traits. In most cases, genes usually exist in the form of nucleotide sequences. In a cell, the DNA sequence of a gene is first transcribed into mRNA, which serves as the template for protein translation. The newly synthesized proteins contribute to biological processes in an organism. To understand the biochemical, biophysical, and genetic functions of a given gene and its protein, recombinant DNA technologies have been developed and used extensively. Since 1970s, the discovery of restriction enzymes has facilitated the development of molecular cloning methods and allowed the manipulation of DNA sequences selectively and specifically to create novel recombinant molecules[1]. DNA fragments are inserted into vectors to form recombinant genetic materials for their replication, studies of gene functions and productions of recombinant proteins. The recombinant DNA technology was first used to study gene functions when the genes responsible for metabolism of galactose in *E. coli* were fused into the SV40 vector in 1972[2]. For the past few decades, procedures of molecular cloning have been simplified and standardized to construct recombinant DNA with various sizes for different purposes[3]. All these have been applied to generate transgenic organisms and produce recombinant proteins for the use in variety of research and clinical settings.

Glucose enters cells *via* a family of proteins called glucose transporters (GLUTs), which have 14 known members. Glucose transporter 4 (GLUT4) encoded by *SLC2A4* gene in human genome and *Slc2a4* gene in others such as rodent genomes has 409 amino acid residues, and a K_m value of 5 mmol/L for glucose[4]. GLUT4 was first identified in a screen for the insulin-stimulated glucose transporter in cell membrane preparations of rat adipocytes using monoclonal antibodies against these membrane proteins[5]. Subsequently, *Slc2a4* gene was cloned from rat adipose tissue, and is homologous with GLUT1, which is encoded by *Slc2a1* gene[6-8]. GLUT4 is expressed in not only adipose and muscle cells, but also other tissues such as the heart and brain[9]. The N- and C- termini of GLUT4 are located in the cytoplasm and responsible for the insulin-mediated translocation from the cytosol to the cell membrane [10]. The current model is that insulin stimulates GLUT4 translocation from the intracellular locations to the plasma membrane, where it facilitates the glucose entry into cells[11]. In addition, exercise also stimulates the expression of *SLC2A4* mRNA in the skeletal muscle and improves insulin sensitivity in human patients[12], which may be mediated by GLUT4[13]. Insulin-stimulated glucose transport is significantly impaired in the skeletal muscle of patients with type 2 diabetes[14]. Therefore, understanding the role of GLUT4 in the regulation of glucose homeostasis is critical for the prevention and treatment of type 2 diabetes.

Here, we summarize the recombinant DNA technologies used to study expression profiles and functions of GLUT4 in tissues and cells. Key words as indicated in the following sections were used to search PubMed. The title and abstracts of the retrieved articles were read by authors. Only the articles that contained descriptions of knockout, transgenic overexpression and knockdown molecular techniques, and confirmed gene or protein expression levels were chosen for further reading. The methods used to manipulate the expression levels of GLUT4 *in vivo* and *in vitro* and reported observations in retrieved studies were summarized here. This review may help researchers who are interested in the physiological functions of GLUT4 to have a clear understanding of the status.

THE COMMON MOLECULAR BIOLOGY TECHNIQUES TO STUDY GENE AND PROTEIN FUNCTIONS

The development of molecular cloning techniques allows isolation, generation, and production of DNA sequence independence of the species and organisms that carry the original sequences. DNA fragments isolated from genomes or created *via* polymerase chain reaction (PCR) are inserted into vectors that can replicate and express in the host cells, and in turn alter the genetic features of the host cells, tissues or organisms[15]. PCR technique quickly produces large numbers of copies of a specific DNA fragment for sequencing analysis and molecular cloning. Cloning of a specific DNA sequence helps to explore the gene's biological functions, and to create large amounts of protein, such as growth hormone, insulin and clotting factors for therapeutic purposes[16]. In addition, a comparison of DNA sequences from different organisms can determine the evolutionary relationship within and between species, and functional domains of a gene. Recombinant DNA technologies can be used in gene therapies to treat diseases such as immunodeficiency diseases and metabolic disorders[17] and diagnosis of genetic diseases[18]. Genetically modified organisms or genetically engineered organisms can be created *via* alterations of the genetic sequences of the chromosome or insertions of the foreign DNA fragments into the genome to alter the phenotypes of the offspring[19].

Genes in plants, animals and microorganisms have been deleted or their expression levels have been knocked down to investigate their functions or treat genetic diseases clinically[20]. Methods are developed to silence or remove the target gene, such as gene silencing, conditional knockout, homologous recombination, and gene editing[20]. Homologous recombination occurs when homologous recombinases (nucleases) recombine two linearized DNA fragments with the same terminal sequences to create a novel fragment for molecular manipulations[21]. This makes accurate gene editing possible and becomes emerging tools in genetics[22]. Zinc finger nucleases, transcription activator-like effector nucleases, and clustered regularly interspaced short palindromic repeat (CRISPR) are developed and shown differences in knockout efficiency, completion time, and off-target efficiency[20]. Each of these techniques uses a nuclease to introduce DNA double-strand breaks at the targeted locations with the guidance of homologous binding proteins or RNA[23]. Gene knockdown methods such as RNA-based RNA interference, small interfering RNA and short hairpin RNA (shRNA), and antisense oligonucleotides have been developed to inhibit protein expression[24]. RNA interference (RNAi) is triggered by double-stranded RNA and causes the sequence-specific mRNA degradation of the single-stranded target RNA[25]. Small non-coding RNA molecules can also act to inhibit RNA translation[26].

In addition to the change of gene expression, tagged proteins or fusion proteins with novel properties can be created using molecular biology tools[27]. Fusion or tagged proteins with two or more domains from different proteins can be easily obtained and purified for their uses in research and clinical treatments, detection of the expression levels, and visualization of the intracellular locations of the expressed proteins[27]. These have been used to create vaccines, multifunctional enzymes, targeted drugs, thrombolytics, antimicrobial peptides, *etc*[28].

MOLECULAR BIOLOGY TECHNIQUES USED IN THE STUDIES OF GLUT4

The identification of GLUT4 and cloning its gene[29] have facilitated the studies of its tissue distribution, functions, the mechanisms responsible for its translocation, and the regulations of its protein and mRNA expressions in different cells. The tagged or fluorescent GLUT4 fusion proteins are used to study its intracellular trafficking. GLUT4 overexpression and knockdown, and *Slc2a4* gene knockout *in vitro* and *in vivo* have been developed to study the insulin-stimulated GLUT4 translocation and glucose homeostasis, which contribute significantly to our understanding of the role of GLUT4[29]. To review the techniques of molecular biology in the study of GLUT4, "GLUT4, molecular biology" and "*SLC2A4*, molecular biology" as keywords were used to search the PubMed database to retrieve relevant articles. We have focused on the techniques used in *Slc2a4* knockout, knockdown and transgenic studies, and results associated with the genetic changes *in vivo* and *in vitro* were analyzed and summarized here. As shown in **Figure 1**, *Slc2a4* genes have been knocked out and GLUT4 protein has been overexpressed in the whole body and in specific tissues and cells. In addition, GLUT4 protein has been knocked down using shRNA, and its translocation has been studied using fusion or tagged proteins. Various methods such as *in situ* hybridization, fluorescent microscopy, immunohistochemistry, Western blotting for protein and Northern blot and real-time PCR for mRNA used to determine endogenous or transgenic GLUT4 expressions are also summarized in this review.

Whole-body and tissue specific *Slc2a4* knockout studies

Slc2a4 mRNA expression is detected not only in brown and white adipose tissue, skeletal and cardiac muscle, but also in other tissues such as neurons[30]. To study GLUT4 functions, mice with the *Slc2a4* deletion in the whole body or specific tissues or cells have been created. We searched PubMed to retrieve the original articles that initially reported the *Slc2a4* deletions. **Table 1** shows the techniques for creating knockouts, experimental animals, methods to confirm the gene deletion and expression, and

Table 1 Methods used to create whole body and tissue specific *Slc2a4* knockout animals, tissues and animals studied, analytic methods included, and observations reported

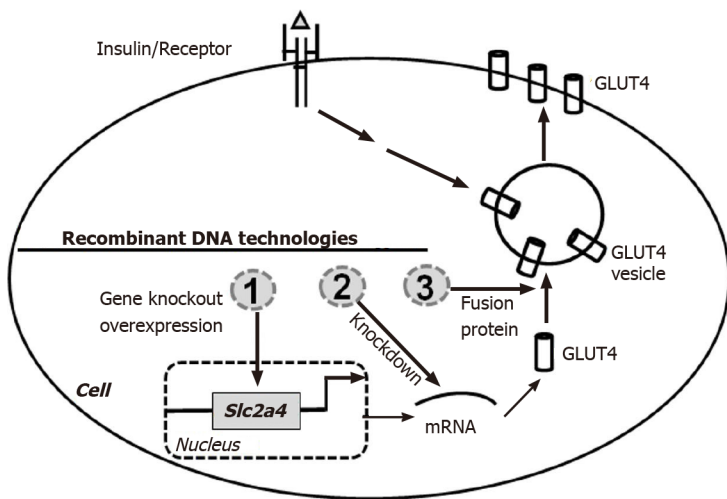
Methods	Tissues/Animals	Analysis	Observations	Ref.
A construct with a disrupted mouse <i>Slc2a4</i> gene was electroporated into WW6/22 ES cells to create deletion, which were microinjected into C57Bl/6 blastocysts	Skeletal muscle/GLUT4-null mice and wild-type control mice	Southern blot for DNA, Northern blot for mRNA, and Western blot for protein measurements	The <i>Slc2a4</i> ^{-/-} mice have normal glycemia, growth retardation, decreased longevity, cardiac hypertrophy, reduced adipose deposits, postprandial hyperinsulinemia, and lowered insulin sensitivity; The male <i>Slc2a4</i> ^{-/-} mice have lower and higher blood glucose levels than the controls in fasted and fed states, respectively	[31]
GLUT4-loxP mice were crossed with α -MHC promoter-driven Cre	Heart/Cardiac-selective <i>Slc2a4</i> ^{-/-} deletion mice (G4H ^{-/-} mice) and control mice	Southern blotting and PCR for DNA, and Western blot for GLUT4 levels using various antisera	G4H ^{-/-} mice have modest cardiac hypertrophy, normal life span and serum levels of insulin, glucose, FFAs, lactate, and β -hydroxybutyrate, increased basal cardiac glucose transport and GLUT1 expression, and abolished insulin-stimulated cardiac glucose uptake	[33]
GLUT4loxP mice as shown in [33] were crossed with the muscle CK promoter driven Cre transgenic mice to obtain Muscle-G4KO	Skeletal muscle/Muscle-G4KO mice and heterozygous <i>Slc2a4</i> deletion mice in the 129SV and C57Bl/6J background	Reverse transcription-PCR for mRNA, and Western blot for GLUT4 protein (anti-GLUT4 AB1346)	Muscle-G4KO mice show a reduction in basal and near-absence of insulin- or contraction-stimulated glucose transport, showing; severe insulin resistance and glucose intolerance from an early age	[34]
GLUT4-null mice were crossed with transgenic mice expressing GLUT 4 driven by MLC promoter[55] to create MLC-GLUT4-null mice	EDL and soleus muscle/MLC-GLUT4-null mice having GLUT4 in the fast-twitch EDL muscle, GLUT4 null mice, and control mice	Western blot for GLUT4 protein (rabbit polyclonal antiserum)	MLC-GLUT4-null mice have less GLUT4 in WAT (females only) and soleus muscle, adipose tissue deposits, adipocyte size, and plasma free fatty acid levels in the fed state than the controls. Glucose uptake in the EDL, but not in the soleus, muscle is restored to normal in male and above normal in female MLC-GLUT4-null mice	[32]
GLUT4-loxP mice were crossed with aP2-driven Cre transgenic mic to obtain G4A ^{-/-} mice	Adipose tissue/G4A ^{-/-} , and control mice	Western blot for GLUT4 protein in BAT and WAT tissues	G4A ^{-/-} mice show impaired insulin-stimulated glucose uptake in adipocytes, glucose intolerance, hyperinsulinemia, and insulin resistance in the muscle and liver	[35]
The G4A ^{-/-} mice[35] were crossed with the muscle-G4KO mice[34] to generate AMG4KO mice	Adipose tissue and skeletal muscle/G4A ^{-/-} , muscle-G4KO, and AMG4KO mice	Western blot for GLUT4 protein using antibodies from H. Haspel in the Charles River Laboratory	AMG4KO mice develop fasting hyperglycemia and glucose intolerance and are at risk for greater insulin resistance than mice lacking GLUT4 in only one tissue	[37]
The neuron-specific Nestin promoter-driven Cre transgenic mice were crossed with GLUT4-loxP mice (FVB strain) to obtainBG4KO mice	Whole brain/BG4KO and control mice	Western blot for GLUT4 protein in the brain using antibody from Chemicon	BG4KO mice have glucose intolerance, insulin resistance, and impaired glucose sensing, suggesting that the brain GLUT4 may sense and respond to glucose	[36]

α -MHC: α -myosin heavy-chain; AMG4KO: Adipose/muscle-GLUT4 double knockout; BAT: Brown adipose tissue; BG4KO: Brain-specific GLUT4 knockout; Cre: Cre recombinase; CK: Creatine kinase; ES: Embryo stem; EDL: Extensor digitorum longus; GLUT4: Glucose transporter 4; G4A^{-/-}: Adipose tissue-specific GLUT4 knockout; GLUT4-loxP: *Slc2a4* allele with exon 10 flanked by loxP sites; G4KO: GLUT4 knock out; MLC: Myosin light chain; Ref: References; WAT: White adipose tissue.

observations. In the end, seven representative articles that the research groups generated a specific knockout model to study GLUT4 and clearly described the methods of GLUT4 deletion are summarized here as shown in Table 1. The animal models were also used by many other groups.

In 1995, the mouse *Slc2a4* locus was disrupted using homologous recombination in embryonic stem cells which generated mice without GLUT4 expression (GLUT4-null) in the whole body[31]. The GLUT4-null mice showed growth retardation, enlarged hearts and complete lack of the white adipose tissue[31]. GLUT4-null mice have normal glucose tolerance and basal glucose turnover rates. However, they are insulin intolerant, suggesting insulin resistance. Later on, the GLUT4-null mice[31] have been used to create mice expressing GLUT4 specifically in the extensor digitorum longus muscle[32].

Tissue specific GLUT4 knockout mice have been created by crossing mice carrying a *Slc2a4* allele with exon 10 flanked by loxP sites with those carrying Cre gene expression driven by tissue specific promoters[33-36]. The various phenotypes of these knockout mice help us to understand the roles of GLUT4 in different tissues and glucose metabolism. For example, the muscle-specific GLUT4 knockout mice (muscle-G4KO) were created by breeding mice carrying the *Slc2a4* exon 10 flanked by loxP sites with mice carrying a transgene encoding Cre recombinase under the control of the muscle creatine kinase promoter[34]. Compared with GLUT4-null mice, muscle-G4KO mice have normal body weight and fat pad weight at least before 6 mo of age[34]. The skeletal muscle mass is also normal. The increase in heart weight is consistent with GLUT4-null mice[31] and cardiac-G4KO mice[33]. Compared with the shortened lifespan of GLUT4-null mice, the life span of muscle-G4KO mice is normal. In contrast to GLUT4-null mice and cardiac-G4KO mice, adipose-G4KO mice[35] are similar to muscle-G4KO mice.



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Figure 1 Recombinant DNA technologies used in the study of glucose transporter 4 functions and its translocation mechanism. *Slc2a4* gene is transcribed into mRNA, which is translated into glucose transporter 4 (GLUT4) protein. The binding of insulin to its receptor leads to the activation of insulin signaling system, which facilitates the movement of GLUT4 from its intracellular location to the cell membrane, and in turn the entry of glucose in the cells. Recombinant DNA technologies have been used to alter gene expression level (1), reduce mRNA translation (2) and tracing intracellular movement of GLUT4 protein (3). 1. *Slc2a4* gene has been deleted in the whole body via homologous recombination and in individual tissues or cells via Cre-LoxP system driven by tissue specific promoters. In addition, transgenic overexpression of GLUT4 in whole body or specific tissues and cells has been done using mini gene or *SLC2A4* cDNA driven by different promoters, respectively. 2. The GLUT4 protein is knocked down using short-hairpin RNA under the control of different promoters to interfere the translation process. 3. Fusion or tagged GLUT4 has been created to study the insulin-stimulated GLUT4 translocation mechanism using fluorescent microscopy, and immune assays.

However, adipose-G4KO mice have glucose intolerance[35]. Interestingly, unlike other GLUT4 knockout mice, the heart weight of adipose-G4KO mice is normal.

In addition, adipose and muscle *Slc2a4* double knockout (AMG4KO) mice are also created by crossing the respective tissue knockout mice[37]. Interestingly, these AMG4KO mice develop hyperglycemia in the fasting state[37]. It appears that GLUT4 also plays a role in a physiological condition that does not need the insulin-stimulated glucose uptake.

GLUT4 knockdown studies

The deletion of a gene completely stops the genetic information flow. Another way to block the protein expression is to knockdown a gene's expression, which temporarily stops or reduces the expression of the targeted gene. Unlike knockout, gene knockdown involves the methods interfering with RNA molecules (mRNA or non-coding RNA) that bridges DNA and proteins. "GLUT4, knockdown" and "SLC2A4, knockdown" as keywords were used to search the PubMed database to retrieve relevant articles. Table 2 summarizes the methods of knockdown and confirmation, cells used, observations of GLUT4 knockdown studies.

Recently, RNAi has emerged as a powerful tool for the study of gene function in mammalian cells [38]. After transfection, the shRNAs molecules are transcribed under promoters in constructs that drive the RNA synthesis within the targeted cells. Oligo nucleotides with sequences of shRNAs may be transfected directly into the cells[38]. In all studies summarized in Table 2, shRNAs method is used to achieve GLUT4 knockdown[39-41], which is delivered *via* recombinant retroviruses. Two of three studies investigated the roles of GLUT4 in 3T3-L1 adipocytes. It appears that GLUT4 expression is needed for the lipogenesis after differentiation in 3T3-L1 cells, but not necessary for lipogenesis during differentiation[41]. In addition, the insulin-regulated aminopeptidase trafficking is not always associated with the GLUT4 movement[40].

Transgenic studies

We have used key words "GLUT4 transgenic" (314 hits) and "GLUT4 overexpression" (609 hit) to search PubMed to retrieve GLUT4 transgenic studies. After going through the titles or abstracts containing "GLUT4 overexpression and GLUT4 transgenic", we found 15 papers that have described their original methods or clearly cited the methods used by them, confirmed the GLUT4 overexpression in mice and provided results. Table 3 summarizes the techniques used to overexpress GLUT4, the methods to confirm the expression and results observed in those animals.

In 1992, a 2.4-kb fragment of 5' flanking DNA of human *SLC2A4* promoter fused with the bacterial chloramphenicol acetyltransferase (CAT) as a reporter construct was developed and used to show the *SLC2A4* expression profile in mice[42]. In 1993, an 11.5-kb *SLC2A4* mini gene in pHSS6 vector was created and used to overexpress human GLUT4 in the whole body of mice[43]. As shown in Table 3, the

Table 2 Methods used to knockdown glucose transporter 4 and analyze its expression in cell lines, and reported observations

Methods	Cells	Analysis	Observations	Ref.
Recombinant lentivirus was used to express shRNA based on <i>SLC2A4</i> sequence (NM_001042.3)	Human head and neck squamous cancer cell lines, HSC-2	Western blot for GLUT4 protein using antibody from Epitomics	The knockdown of GLUT4 expression in HSC-2 cells induced DDX58 and OASL protein expressions, and reduced cell migration in culture	[39]
pSIREN RetroQ system was used to obtain recombinant retroviruses that produce shRNAs corresponding to mouse <i>Slc2a4</i> sequence GGTGATTGAACAGAGCTAC (GenBank ID was not provided)	3T3-L1 adipocytes	Immunofluorescence of phase-contrast and epifluorescence images for GLUT4 protein using antibodies (rabbit anti-GLUT4, a gift from Dr. Sam Cushman (National Institutes of Health))	GLUT4 knockdown does not affect IRAP trafficking, showing that IRAP traffics is independent of GLUT4	[40]
Recombinant lentivirus was used to generate shRNA under the control of human H1-RNA promoter using the mouse <i>Slc2a4</i> mRNA sequence (GenBank ID not provided)	3T3-L1 adipocytes	Immunofluorescence microscopy and Western blot for GLUT4 using rabbit polyclonal antibody from Chemicon International Inc	GLUT4 knockdown in 3T3-L1 adipocytes reduces insulin-stimulated glucose uptake by 50%-60%, IRAP expression of depending on differentiation stage, and lipogenic capacity of differentiated, but not differentiating cells	[41]

DDX58: DEXD/H-Box Helicase 58; GLUT4: Glucose transporter 4; IRAP: Insulin-regulated aminopeptidase; OASL: 2'-5'-Oligoadenylate Synthetase Like; Ref: References; shRNA: Short hairpin RNA.

mouse line containing the 11.5-kb mini gene has been used in seven out of eight papers testing the effects of overexpression of human GLUT4 in the mouse whole body on metabolism[43-49]. CAT activity, *SLC2A4* mRNA and/or GLUT4 protein level in adipose tissue and skeletal muscle and other tissues have been analyzed to confirm the success of transgenic expression[43,44]. In general, the whole-body human GLUT4 overexpression reduces blood glucose in both fasting and fed states and increased glucose uptake in mice, but affects the blood insulin level in wild type mice and diabetic mice differentially[44]. Whole body GLUT4 overexpression does not alter body weight, but can reduce blood glucose level and affect serum insulin level.

GLUT4 has been overexpressed in a tissue specific manner as shows in Table 4. Majority of the studies have been focused on adipose tissues[50-54]. There is one for the skeletal muscle[55] and one for adipocytes[56]. A 6.3-kb genomic DNA fragment of human *SLC2A4* gene driven by the mouse *ap2*[50] promoter has been used to overexpress GLUT4 in mouse adipose tissues. This method was first published in 1993[50] and was used in many other studies in the genetic settings of wild-type and diabetic mice[51-54]. Like the papers summarized in Table 3, a human *SLC2A4* gene under a tissue-specific promoter was used to overexpress GLUT4. Human *SLC2A4* cDNA was also used to overexpress GLUT4 in adipocytes[56], but mouse *Slc2a4* gene was used to express GLUT4 in the hindlimb muscle [55]. All adipose tissue specific GLUT4 overexpression studies[50-55] tested the *SLC2A4* mRNA or GLUT4 protein level to confirm GLUT4 expression, which is found to be expressed in both brown and white adipose tissues. In conclusion, adipose tissue specific GLUT4 overexpression in mice can cause increases in body and adipose tissue weights[50,51]. The adipose tissue specific GLUT4 transgenic mice also have higher glucose disposal rate, may be caused by increased basal and insulin-stimulated glucose transport rate. It is interesting to find out that the elevated expression of GLUT4 in the adipose tissue only can increase glucose transport rate and adipose tissue weight, which is associated with the significant increase in body weight, suggesting the importance of GLUT4 expression in adipose tissue.

CONCLUSION

Conclusion and future perspectives

As summarized in this review, methods such as whole body and tissue specific gene knockout, recombinant viruses, real-time PCR, immunofluorescence, stable cell line and transgenic animals have been used to study GLUT4 system and insulin action in different target cells and tissues. The advantages of using multiple molecular biology methods allow us to confirm the functions of GLUT4 for insulin-stimulated glucose transport in different cells and tissues, and in the regulation of whole-body glucose homeostasis. Interestingly, GLUT4-null mice which do not have a functional *Slc2a4* gene in the whole genome have normal glucose tolerance and basal glucose turnover rates, but they are insulin-intolerant which suggests insulin resistance[31]. AMG4KO mice (adipose and muscle double knockout) have reduced whole body glucose uptake and hyperglycemia[37]. Compared with GLUT4-null mice, AMG4KO mice have more severe glucose homeostasis defects. Although the explanation for this difference is not clear, differences in genetic background and differences in developmental stages, where GLUT4 is deleted have been proposed. More importantly, the hyperglycemia in these double

Table 3 The transgenic studies using the *SLC2A4* mini gene and its promoter for the whole-body expression in mice

Transgenic constructs	Analysis	Observations	Ref.
A 11.5-kb mini gene of human <i>SLC2A4</i> starts with a 5.3-kb fragment upstream of transcription start and terminates within exon 10 of the gene followed by the bacterial CAT in pHSS6 vector	RNase protection assay and Western blot were used for <i>SLC2A4</i> mRNA and GLUT4 protein in BAT, WAT, heart and skeleton muscle, respectively	The transgene expression was detected in WAT and BAT, heart and skeleton muscle of mice. Female transgenic mice have higher GLUT4 protein in the adipose tissue and less <i>SLC2A4</i> mRNA in skeleton muscle than male ones. Transgenic mice have higher GLUT4 protein level in adipose tissue, liver, heart and skeleton muscle than the controls	[43]
The 11.5-kb minigene with the CAT reporter as shown in[43]	Reverse transcription PCR was used to measure <i>SLC2A4</i> mRNA in cardiac and hindquarter muscle, BAT and WAT. Immunofluorescent test was for GLUT4 translocation	Transgenic mice gained more weight after 15 wk old of age, and have lower blood glucose in both fasting and fed states, lower insulin level in fasting and higher after refeeding, and higher glycogen contents, GLUT4 translocation in cardiac and skeleton muscle than the control mice	[44]
The 11.5 kb minigene with the CAT reporter as shown in[43]	Western blot was used to detect GLUT4 in gastrocnemius muscles	Transgenic mice have lower serum glucose level in both fasting and fed state, higher insulin level during fasting and lower after fed than the control ones	[45]
The 11.5 kb minigene with the CAT reporter as shown in[43]	Western blot was used to detect GLUT4 in the heart	Transgenic mice have higher glucose uptake, glycolysis and glycogen content, and lower insulin-stimulated glycolysis rate and glycogen synthesis in the heart than the control ones. Glucose and fatty acid oxidation remain the same	[46]
The 11.5 kb minigene with the CAT reporter as shown in[43]	Immunofluorescence was used to detect GLUT4 in cardiac myocytes and adipocytes	Transgenic mice have similar body weight, and epididymal adipose tissue weight and adipocyte size as the controls. Transgenic mice have higher levels of triglycerides, β -hydroxybutyrate and free fatty acids, and parametrial fat weight and lower glucose level after an oral glucose challenge and insulin level after an insulin injection than the controls. The insulin-stimulated glucose uptake is impaired in transgenic mice	[47]
A 2.4-kb of 5' flanking DNA fragment of human <i>SLC2A4</i> promoter fused with the CAT as a reporter construct	CAT activity assay and RNase protection assay were used to detect promoter activation and mRNA, respectively	In transgenic mice, CAT activity can be detected in the tissues that generally express GLUT4, including BAT and WAT, and smooth, skeleton and cardiac muscle, but not the liver	[42]
A 2.4-kb of 5' flanking DNA of human <i>SLC2A4</i> promoter fused to CAT as shown in[42]	Western blot was used to detect GLUT4 in adipose and skeleton muscle tissues	Transgenic mice have slower rise of blood glucose (no difference in glucose and insulin levels) during pentobarbital sodium anesthesia, and higher glucose infusion rate (40% increase) during hyperinsulinemic euglycemic clamp than the controls	[48]
A 2.4-kb of 5 flanking DNA of human <i>SLC2A4</i> promoter fused to CAT as shown in[42]	Only cited previous publications[42]	Transgenic mice have lower blood glucose, higher lactate and β -hydroxybutyrate levels during both fasting and fed states, and better glucose transport in the soleus muscle when fed a high-fat and high-sugar diet than the controls	[49]

BAT: Brown adipose tissue; CAT: Chloramphenicol acetyltransferase; GLUT4: Glucose transporter 4; Ref: References; WAT: White adipose tissue.

knockout mice develops in the fasting state, rather than fed state[37]. This phenomenon appears to indicate that GLUT4 plays an important role in the control of glucose homeostasis during fasting, a state that insulin level is low. The translational value of these observations is that GLUT4's physiological role from the integrated homeostatic point of view may be extended beyond the insulin-stimulated glucose uptake. Of course, more studies are warranted on this line of research.

On the other hand, the GLUT4 knockdown studies used the shRNAs method and have been done in cell lines to reduce GLUT4 expression. This may be helpful for us to understand the GLUT4 functions and the underlying mechanisms in particular cells. It appears that GLUT4 expression is not necessary for lipogenesis during 3T3-L1 cells differentiation. Apparently, it will be helpful when more GLUT4 knockdown studies are done in animals.

The GLUT4 overexpression in transgenic mice at wholebody level reduces blood glucose in both fasting and fed states and increased glucose uptake, glycolysis and glycogen level[44]. Compared to the control mice, overexpression of GLUT4 in adipose tissue in mice leads to lowered blood glucose in the fasting state, and increase in body weight and adipose tissue weight[50]. The expression of GLUT4 in adipose tissue and skeleton muscle affects the rate of whole-body glucose disposal, which may be caused by increased basal and insulin-stimulated glucose transport rates. This lowered blood glucose level in the transgenic mice also indicates that GLUT4 probably plays a role in the basal glucose uptake.

For the tissue specific GLUT4 knockout, Cre-loxP-mediated gene recombination under the control of promoters has been the main method to delete *Slc2a4* gene. Since the development of CRISPR technology, it has not been used to knockout *Slc2a4* in whole body or tissues, which is a limitation in the field. We have used "GLUT4" and "CRISPR", and "*SLC2A4*" and "CRISPR" as key words to search PubMed, and retrieved eight and two published articles, respectively. However, none of the published articles used the CRISPR methods to knockout *SLC2A4* or *Slc2A4* gene in cells or animals. All of them used CRISPR methods to study the components in the exocytosis process of GLUT4 translocation. As

Table 4 Recombinant DNA techniques to create tissue specific glucose transporter 4 overexpression in animals and cells, analysis performed, and observations reported

Techniques	Tissue/analysis	Observations	Ref.
A 6.3-kb genomic DNA fragment of human <i>SLC2A4</i> gene is under the control of a 5.4-kb DNA fragment of mouse ap2 promoter using Gateway cloning	Adipose-specific overexpression/ Western blot was used to detect GLUT4 in BAT and WAT	Transgenic mice have lower glucose level in the fasting, insulin level in the fed state, higher body weight and body fat at 18 to 21 wk of age, and higher basal and insulin-stimulated glucose transport rates in epididymal, parametrial, and subcutaneous adipocytes than the controls	[50]
Same as in [50]	Adipose-specific overexpression/Only cited previous publications[50]	Transgenic mice have higher body weight, parametrial fat pad weight and adipocyte size, and glucose transport in both fasting and fed states, and lower plasma insulin and glucose levels after a glucose challenge than the controls	[51]
Same as in [50]	Adipose-specific overexpression/Only cited previous publications[50]	Transgenic mice have higher glucose disposal rate in a glucose tolerance test, and palmitic acid-hydroxy stearic acid levels in serum, WAT and BAT than the controls	[52]
Same as in [50]	Adipose-specific overexpression/Western blot was used to detect GLUT4 in BAT and WAT	Transgenic mice fed a high-fat diet have higher glucose disposal rate than those fed a low-fat diet, and stable GLUT4 expression in fat and no increase in body fat	[53]
Same as in [50]	Adipose-specific overexpression/Western blot was used to detect GLUT4 in BAT and WAT	Transgenic mice have higher gonadal adipose weight, basal and maximum insulin stimulated glucose transport in isolated adipocytes, glucose transport rate, triglyceride synthesis and CO ₂ production than the controls	[54]
A 4.5-kb DNA fragment of the mouse <i>Slc4a2</i> gene is under the control of a 3-kb fragment of the mouse myosin light chain gene promoter	Hindlimb muscle overexpression/Northern blot and Western blot were used to detect <i>Slc4a2</i> mRNA and GLUT4 in different tissues respectively	Transgenic mice have higher basal and insulin-stimulated glucose uptake and turnover, higher glycogen content in the skeleton muscle, higher insulin sensitivity, higher levels of free fatty acid and ketone in both fasting and fed state, and lower fasting glucose level than the controls	[55]
The human <i>SLC2A4</i> cDNA is driven by the CMV promoter in pCIS2 vector	Rat adipocytes overexpression/Immunofluorescence was used to detect GLUT4 overexpression	Rat adipose cell transfected with the GLUT4 construct had significantly higher antibody binding after insulin stimulation than the control cells	[56]

BAT: Brown adipose tissue; CMV: Cytomegalovirus; GLUT4: Glucose transporter 4; Ref: References; WAT: White adipose tissue.

CRISPR has been developed and used widely, GLUT4 knockout/knockdown through this system may be worth to be done. This may provide us another tool to manipulate the GLUT4 expression in the whole body or in tissue specific manners.

In addition, results of glucose tolerance are different between mice with whole body and tissue specific GLUT4 knockout. Therefore, whether the loss of GLUT4 in a specific tissue (muscle or fat) or the expression of GLUT4 in other tissues without gene deletion plays a role in this difference is worth to be investigated. It is safe to say that more research works are anticipated in the future to precisely define the role of GLUT4 in the control of glucose homeostasis at whole body and tissue levels. In so doing, we develop effective ways to prevent and treat type 2 diabetes mellitus.

FOOTNOTES

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